Characterization of the Major Sialyl-Le\^a-positive Mucins Present in Colon, Colon Carcinoma, and Sera of Patients with Colorectal Cancer\^1

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ABSTRACT

The expression of the mucin-bound sialyl-Lewis\^a epitope is increased in the tissue of most colorectal carcinomas and in the sera of about 30% of tumor patients. In colon cancer, a portion of the sialyl- Le\^a groups detectable with the monoclonal antibody AM-3 is located on MUC1 (C. Hanski et al., Cancer Res., 53: 4082-4088, 1993). In order to characterize the major colon carcinoma-associated sialyl- Le\^a-positive glycoprotein components, the tissue- and serum-derived antigens were investigated. The buoyant densities of the sialyl-Lewis\^a-positive antigens from tumor and normal colonic tissues and from sera of patients with colon carcinoma and healthy donors correspond to that of mucins (1.40 g/ml). The sialyl- Le\^a-positive mucins purified from both tissue clusters under nonreducing conditions in the void volume of a Sephacrose CL-2B column, indicating a molecular mass more than 2 \times 10^7 daltons. They yield in immunoblot after SDS gel electrophoresis under reducing conditions a main band at an apparent Mr 880,000. Radioactive labeling revealed that the band of Mr 880,000 is the major protein component in sialyl-Lewis\^a-positive mucins both from tumor and normal colonic tissue. In sera of colon carcinoma patients, the sialyl- Le\^a glycoprotein is also detectable mainly on a Mr 880,000 glycoprotein band and, additionally, on a Mr 140,000 molecule as well as on \alpha\^-acid glycoprotein. Sera from healthy donors exhibited only a sialyl- Le\^a-positive glycoprotein with the apparent Mr 140,000. Sandwich ELISA as well as immunoblots of mucins purified from the colon carcinoma cell line LS174T indicated that the sialyl- Le\^a glycoprotein migrating in the Mr 880,000 band is located on MUC2 protein core. Together, these data suggest that sialyl- Le\^a antigen in colon, colon carcinoma, and the sera of patients with this tumor is located on the MUC2 molecule, consisting of several subunits with an apparent Mr 880,000, linked via disulfide bridges. The increase of sialyl- Le\^a expression in colon carcinomas appears to be mainly due to a more frequent transfer of sialyl- Le\^a moieties onto the mucin core in tumor tissue.

INTRODUCTION

Altered blood group antigens represent a family of carbohydrate epitopes frequently overexpressed in malignant tissues (1–3). The overexpression of the carbohydrate structure sialyl- Le\^a was detected in 76% to more than 90% of colon carcinomas by immunohistochemistry with the mAb CSLEX-1 (4) and the mAb AM-3 (5). The related antigen sialyl- Le\^a, defined by the mAb FH6, was found to be overexpressed in 25% of colon carcinomas (6). This latter antigen is detectable in liver metastases on a larger fraction of cells (7), and its expression exceeds that of the primary colon carcinomas (8, 9). These findings led to the conclusion that sialyl- Le\^a may be related to the process of tumor progression (8). Indeed, the expression of sialyl- Le\^a in tumor tissue results in increased concentration of these epitopes in 29 to 35% of patient sera (4, 22–24). The soluble molecules carrying the sialyl- Le\^a structure may modulate the interaction of the blood-borne leukocytes and the endothelial cells. This modulatory role has been postulated for \alpha\^-acid glycoprotein, whose sialyl- Le\^a moieties increase in number during acute phase reaction (25). It has been further shown that the sialyl- Le\^a oligosaccharide alone inhibits neutrophil interaction with endothelial cells and thus suppresses the inflammatory vascular injury (26). Serum molecules carrying sialyl- Le\^a were identified as mucins with a molecular weight > 10^6 in sera of colon carcinoma patients (23). The relationship between the species overexpressed in the tumor tissue and in the sera of colon carcinoma patients has not been investigated in detail.

The purpose of the present work was to obtain more insight into the structure of and the relationship between the tissue and serum-derived colon carcinoma-associated main sialyl- Le\^a-positive molecules. The monoclonal antibody AM-3 was used throughout, which reacts only with the monomer of sialyl Lewis\^a on glycoproteins but not on glycolipids, thus limiting the spectrum of detectable species (27). Furthermore, the question if sialyl- Le\^a overexpression in colon cancer is related to the overexpression of the corresponding mucin protein core was investigated.

MATERIALS AND METHODS

Tissues and Sera. Five carcinomas from sigmoid colon (3) and rectum (2) were separately investigated. Four tumors were moderately (grade 2) and one poorly (grade 3) differentiated; they were in the stage pT2 (2 tumors) or pT3 (3 tumors), corresponding to the Duke's stage A and B, respectively. Two of these patients had metastases to the liver or lymph nodes. Three patients had blood group O, and two patients had blood group A. Sera with high content of sialyl- Le\^a antigen were obtained from five patients with tumors of the rectum (3) or sigmoid colon (2). Four tumors were moderately and one poorly differentiated, in stage pT2 (3 patients) or pT3 (2 patients). Two patients had metastases to lymph nodes, and each of three other patients to liver, skin, or fat tissue. Three patients had blood group O, and two had blood group A.

Purification of Sialyl- Le\^a-positive Mucins from Tissues or Sera. Fresh colonic carcinoma or normal mucosa tissue taken at a distance of 10 cm from the tumor were immediately frozen after surgery and pulverized under liquid nitrogen in a mortar. Tumor and normal tissue from the same patient were always processed in parallel. The powder of 1 g tissue was stirred in 10 ml of buffer containing 4 mM guanidine hydrochloride, 0.1 M Tris, and 5 mM...
EDTA-Na₂ (pH 8.0) for 24 h at 4°C and centrifuged at 100,000 × g for 30 min at 4°C; then the mucins were purified on three CsCl gradients as described by Carlstedt et al. (28). Sera were obtained from colon carcinoma patients prior to surgery or from healthy donors, aliquoted, and kept at −20°C. They were tested for their sialyl-Le⁰ content in sandwich ELISA as described previously (24). Pooled serum samples from five healthy donors or from five patients with colon carcinoma with high sialyl-Le⁰ content were purified in parallel on two CsCl density gradients in the presence of 4 M guanidine hydrochloride.

The third gradient, used to separate DNA from mucins, was omitted in the case of serum. The gradient fractions (1 ml) were collected, and the sialyl-Le⁰ epitope content was determined in ELISA with the AM-3 antibody. The purified, DNA-free, sialyl-Le⁰-positive mucin fractions were further investigated.

**Analytical Gel Chromatography of Sialyl-Le⁰-positive Molecules.** For gel chromatography, purified mucins from tissue or serum were applied to a Sepharose CL 2B or Sepharose CL 6B column (1 × 60 cm) (Pharmacia, Freiburg, Germany) and eluted with 0.1 M Tris-HCI, 5 mM Na₂-EDTA, and 4 M guanidine hydrochloride (pH 8.0). Collected fractions (1 ml) were tested in ELISA for sialyl-Le⁰ content with the AM-3 antibody.

**ELISA.** For determination of the of sialyl-Le⁰ epitope content in CsCl gradient or column eluate, 100 μl of each fraction was bound to Immulon microtiter plates (Dynatech, Denkendorf, Germany) overnight at 4°C, washed, blocked with 1% BSA in PBS, incubated with AM-3 antibody, and detected with a β-galactosidase conjugated anti-mouse IgG as described previously (29). Sandwich ELISA was performed with the BLAST amplification system (Dupont, Dreieich, Germany) with anti-MUC2 antibody 4F1 used at an ascites dilution 1:1000 as catcher and the AM-3-peroxidase conjugate (10 ng/ml) as tracer, as previously described (20).

**Electrophoresis and Immunoblot.** Purified mucins were separated on SDS-polyacrylamide gradient gel (2.5% stacking gel, 3–7.5% running gel). As molecular weight markers, prestained TBA marker (Sigma Chemical Co.) and murine laminin (Collaborative Research, Inc., Bedford, MA) were used. Blotting onto polyvinylidene difluoride membrane (Millipore, Bedford, MA) was carried out in a Trans-Blot apparatus (Bio-Rad, Munich, Germany) at 200 mA for 2 h. Detection of sialyl-Lewis⁰ was performed with the AM-3 antibody as described previously (29). For better detection of MUC1 with the BC3 antibody and MUC2 with 4F1 antibody, the membrane was treated after blotting with a periodate solution (50 mM sodium acetate-100 mM sodium metaperiodate, pH 4.5) overnight in the dark at room temperature as described by Yoo et al. (30).

**Radiolabelling of Purified Mucins.** Two μg of AM-3-positive mucins purified from normal or colon carcinoma tissue of individual patients on three CsCl gradients were iodinated with BioBeads (Bio-Rad) and ¹²⁵Iodine (NEN, Dreieich, Germany; specific activity, 17 Ci/mg) according to the manufacturer’s recommendations. The unbound ¹²⁵Iodine was separated on PD-10 columns (Pharmacia, Freiburg, Germany). The specific activity was 45 × 10⁵ cpm/mg mucin protein.

**Fig. 1.** First CsCl density gradient of tissue extracts and sera. Distribution of sialyl-Le⁰-positive antigens from normal colonic tissue, colon carcinoma, pooled normal human sera, and pooled sera from colon carcinoma patients with high sialyl-Le⁰ antigen content.

**Monoclonal Antibodies.** Monoclonal antibody AM-3 (IgM) has been produced as described previously (5). It is directed against the nonomeric sialyl-Lewis⁰ group (27). Monoclonal antibody BC3 (IgM), kindly donated by Dr. McKenzie (Austin Research Institute, Australia), was raised against human milk fat globule membrane and was shown to react with the variable number of tandem repeats on the MUC1 protein core, the minimal epitope being Ala-Pro-Asp-Thr-Arg (31, 32). The monoclonal antibody 4F1 (IgM) was produced as described previously and is directed against the protein part of MUC2 (33). The monoclonal antibody FH6 directed against sialyl-Le⁰ was kindly donated by Dr. Hakomori (Biomembrane Institute, Seattle, WA). For detection of α1-acid glycoprotein, a rabbit anti-human-α1-acid glycoprotein IgG (Sigma) was used.

**RESULTS**

**Determination of the Bouyant Densities of the Sialyl-Le⁰-positive Antigens from Tissues and Sera.** The distribution of the sialyl-Le⁰-positive antigens in the first CsCl density gradient was similar in extracts from normal and carcinomatous colonic tissues as well as in sera from healthy controls and from colon cancer patients (Fig. 1). The antigens migrate as one band at a density of 1.37–1.41 g/ml (maximum at 1.40 g/ml), characteristic for mucins.

**Determination of the Molecular Weight of the Sialyl-Le⁰-positive Antigens in Tissues and Sera by SDS Gel Electrophoresis and Immunoblotting.** Sialyl-Le⁰-positive mucins were purified from normal colon or colon carcinoma tissue on three CsCl gradients. On immunoblot with the AM-3 antibody after SDS polyacrylamide gel electrophoresis under reducing conditions, the pooled mucin peak showed in sandwich ELISA to carry sialyl-Le⁰ in colon carcinoma (Fig. 2). When mercaptoethanol treatment of the samples prior to electrophoresis was omitted, the major part of the M, 880,000 component remained in the stacking gel (data not shown).

To compare the M, 880,000 band with MUC1, which we recently showed in sandwich ELISA to carry sialyl-Le⁰ in colon carcinoma (20), immunoblot analysis was performed on a purified mucin preparation from colon carcinoma in parallel with the AM-3 antibody and with the anti-MUC1 antibody BC3 (Fig. 3). The AM-3 antibody detected the M, 880,000 band and a very faint additional band at Mr 660,000. In the same or even larger amount of mucins from normal tissue, this band was barely detectable (Fig. 2). When mercaptoethanol treatment of the samples prior to electrophoresis was omitted, the major part of the M, 880,000 component remained in the stacking gel (data not shown).
660,000 and a $M_r$ 400,000 band, indicating the presence of the MUC1 protein core (Fig. 3).

The sialyl-$\text{Le}^\alpha$-positive serum antigens were investigated in parallel. Sera from colon cancer patients or healthy controls migrated in the second CsCl-gradient as a broad band in the density range of 1.25 to 1.42 g/ml. This material obtained from normal serum exhibited in immunoblot a main sialyl-$\text{Le}^\alpha$-positive component with $M_r$ 140,000 (Fig. 2, NS), whereas the sera of colon cancer patients showed the same $M_r$ 140,000 component and, additionally, the high molecular weight component with an apparent molecular weight of 880,000 and a glycoprotein with $M_r$ 44,000 (Fig. 2, TS). In the broad band of the CsCl gradient, two peaks could be discerned: A, maximum at the density 1.39 g/ml; and B, maximum at the density 1.30 g/ml. Immunoblotting of the separated peaks A and B from colon cancer patients sera revealed that peak A contained the $M_r$ 880,000 component and the $M_r$ 140,000 band, while peak B showed only the $M_r$ 140,000 band and the $M_r$ 44,000 band. Normal serum showed in peak A and peak B only the $M_r$ 140,000 band (data not shown).

The gradient fractions were further investigated with anti-human $\alpha_1$-acid glycoprotein, which is known to carry sialyl-$\text{Le}^\alpha$ moieties (34, 35). ELISA analysis of the fractions showed that both sera contained this glycoprotein only in peak B (Fig. 4). This finding was confirmed through pooling of the peak B fractions, separation on 10% SDS-polyacrylamide gel, and immunoblotting with anti-$\alpha_1$-acid glycoprotein-antibody (Fig. 4).

The $M_r$ 44,000 of the single band obtained after immunoblotting of tumor patients’ sera corresponds to the published value of the molecular weight of $\alpha_1$-acid glycoprotein (34).

Analytical Gel Chromatography of Sialyl-$\text{Le}^\alpha$-positive Antigens Purified from Tissues and from Sera. Gel chromatography was performed in order to assess the molecular weight of the major sialyl-$\text{Le}^\alpha$-positive mucins in the native state. Purified mucins from normal colonic tissue and from colon carcinoma tissue were dialysed against elution buffer [0.1 M Tris-HCl, 5 mM EDTA-Na$_2$, and 4 M guanidine hydrochloride (pH 8.0)] and fractionated on a Sepharose
CL-2B column. Mucins purified from either tissue eluted as one peak in the void volume of the column, indicating a molecular mass of more than $2 \times 10^7$ daltons (Fig. 5).

The pooled peak A obtained after the second CsCl gradient centrifugation of serum was also chromatographed on Sepharose CL-2B. The sera of patients with colorectal carcinoma exhibiting a high sialyl-Le$^x$ content revealed high molecular weight components eluting in the void volume of the column (molecular mass more than $2 \times 10^7$ daltons) and, additionally, low molecular weight components, eluting at the end of the included volume (data not shown). Normal serum showed only the low molecular weight peak eluting in the same fractions (data not shown). To obtain a better resolution in the low molecular weight range, the serum-derived material was also chromatographed on a Sepharose CL-6B column. The smaller components still eluted as one peak in the molecular weight range of catalase (232,000), suggesting that the Mr 140,000 band identified in SDS-gel electrophoresis may be a part of a larger molecule composed of subunits bound via disulfide-bridges (Fig. 5).

These data, combined with the results of immunoblotting, indicate that the major portion of the sialyl-Le$^x$-groups present in both tissues is located on at least one mucin molecule with a molecular mass more than $2 \times 10^7$ daltons, composed of subunits with an apparent molecular weight of 880,000 linked via disulfide bridges. Sialyl-Le$^x$-positive mucins exhibiting the same apparent molecular mass of the reduced subunit are also detectable in sera of colon cancer patients but not of healthy individuals. In sera of healthy donors as well as in sera from patients with colon cancer, there is another sialyl-Le$^x$-positive component with the apparent molecular weight of 140,000 and the previously described, sialyl-Le$^x$-positive $\alpha_1$-acid glycoprotein.

**Comparison of the Protein Amount in the M, 880,000 Component of Mucins Purified from Normal or Carcinomatous Colonic**

![Figure 6](cancerres.aacrjournals.org)
Tissue. Mucins purified from 1 g of either tissue on three CsCl gradients were iodinated, separated on a SDS-polyacrylamide gradient gel, and autoradiographed as described. The amount of obtained mucin protein was 170 µg (N) and 130 µg (T). The autoradiography showed one prominent band with an apparent molecular weight of 880,000 and a much weaker band at a molecular weight more than 1,000,000 in both tumor- and normal tissue-derived mucins (Fig. 6, arrowheads). The large amount of radioactivity migrating in the gel front could not be resolved into discrete peptides. In highly concentrated gels it appeared as a homogenous smear (from M₆, 6,000 to 14,000), suggesting partial degradation of the mucin during oxidative ionization reaction (data not shown). The relative amount of the protein in the M₆, 880,000 band in mucins purified from normal or tumor tissue was similar, i.e., there was no overexpression of this protein in tumor tissue. The detection of sialyl-Le⁺ in noniodinated samples from the same preparation by immunoblotting revealed that the sialyl-Le⁺ moiety is several-fold more abundant on the mucin from tumor than from the normal colonic tissue (Fig. 6).

Reactivity of Anti-MUC2 Antibody 4F1 with the M₆, 880,000 Band and Sialyl-Le⁺-positive Mucins. The anti-MUC2 antibody 4F1 (IgM) applied as a catcher in sandwich ELISA selected sialyl-Le⁺-positive mucins from patients' sera as well as from mucins purified from tumor tissue (data not shown), indicating that MUC2 may be present in the material migrating in the M₆, 880,000 band. The ELISA signals were, however, low, and the mucin could not be visualized with the 4F1 antibody on immunoblot. Therefore, sialyl-Le⁺-positive mucins were isolated from LS174T cells, which are known to produce large amounts of MUC2. In this preparation, sialyl-Le⁺ could be detected in sandwich ELISA on the MUC2 protein core and in the M₆, 880,000 band on immunoblot (Fig. 7).

DISCUSSION

One of the striking peculiarities of carcinoma tissue, especially of colon carcinoma, is a very frequent overexpression of the sialyl-Le⁺-bearing glycoprotein, a terminal tetrasaccharide located on glycoproteins and glycolipids. The present work identifies the main mucin carrier of sialyl-Le⁺ in colonic tissue, colon carcinoma, and sera of patients with this tumor. It further suggests that the content of this epitope is greater in tumor as a result of altered glycosylation rather than of the overexpression of mucin protein core. The molecule, with a molecular mass more than 2 x 10⁷ daltons and the reduced subunit with an apparent molecular weight of 880,000, is a major mucin expressed in the normal colonic tissue but, due to the small number of sialyl-Le⁺ moieties, it is barely detectable with AM-3 antibody in immunoblot. It was not detectable in the sera of healthy donors. The appearance of this high molecular weight component in sera of tumor patients may be due to a better detectability and possibly to necrotic processes facilitating the leakage into the circulation.

One of the previously shown sialyl-Le⁺-positive mucins in colon carcinoma is the membrane-located MUC1 (20, 36). The amounts of its protein core are comparable in normal and colon carcinoma tissue, but it is more abundantly modified with sialyl-Le⁺ in cancer (20). The present work shows that MUC1 carries only a minor portion of sialyl-Le⁺ epitopes in the colon carcinoma tissue.

Another well-defined sialyl-Le⁺-positive molecule present in the sera of colon carcinoma patients is α₁-acid glycoprotein. In normal serum, this molecule carries low amounts of sialyl-Le⁺ groups (34), not detectable with the AM-3 antibody in the mucin preparation on immunoblot. The concentration of α₁-acid glycoprotein is known to increase in the sera of patients with colon carcinoma (37). An inflammatory process induces also an increase of the concentration of this glycoprotein and, additionally, of the number of sialyl-Le⁺ groups on the molecule (25). Whether the inflammatory process frequently accompanying tumor growth also enhances the modification of α₁-acid glycoprotein with sialyl-Le⁺ has not been investigated.

Together, these data show that the increase of sialyl-Le⁺ concentration in the sera of colon cancer patients is due to alterations of the concentration and of the extent of modification of several serum glycoproteins, of which the mucin with the apparent M₆, 880,000 subunit is the major one. By contrast, the glycolipids do not contribute to the overall alteration of sialyl-Le⁺ concentration in serum during colon carcinogenesis (38).

Several of the known mucin cores have been detected in colonic tissue and colon carcinoma. Since MUC2 is characterized as the intestinal mucin present in colon (39), the monoclonal antibody 4F1 directed against MUC2 protein core was used to probe the purified sialyl-Le⁺-positive mucins. We detected sialyl-Le⁺ in sandwich ELISA on MUC2 in the sera of tumor patients and in tumor tissue-derived mucins; however, MUC2 could not be visualized in immunoblot with the 4F1 antibody. If the same experiments were repeated with mucins purified from LS174T cells, the presence of sialyl-Le⁺ on MUC2 molecule and its apparent molecular weight of 880,000 after reduction could be demonstrated. Although LS174T-derived mucins seem to be less glycosylated and to have better accessible MUC2 moieties, it is barely detectable with AM-3 antibody in immunoblot. The autoradiography after iodination of purified sialyl-Le⁺-positive mucins from normal and tumor tissue showed that the material contained the major 880,000 band and a minor band at a molecular weight more than 1,000,000. It appears, therefore, that the amounts of other mucins copurifying with the sialyl-Le⁺-positive mucins are low. Due to a very limited separating capacity of polyacrylamide gels in the high molecular weight range, however, it cannot be excluded that mucins with other protein cores than MUC2 also migrate at the same apparent molecular weight.

In previous studies, sialyl-Le⁺Le⁺ epitope was detected with the FH6 antibody in colon carcinoma tissue and in colon carcinoma-derived metastases on a mucin-like glycoprotein with a molecular weight of more than 1,000,000 (7, 9). Since the FH6 antibody detects, in our preparations, only the M₆, 880,000 band, it is possible that one of the previously reported glycoproteins is identical with the sialyl-Le⁺-positive MUC2 described in the present work, the differences in molecular weight being due to the different electrophoretic conditions.

Several leukocyte mucin molecules have been identified recently as ligands to P- and L-selectin (reviewed in Ref. 40). These heavily...
O-glycosylated, membrane located glycoproteins stretch far beyond the glycoalxay layer and are the primary targets for interaction with the cell environment. The MUC2 molecule appears to be one of the carriers of sialyl-Lewis epitopes present on normal and malignantly transformed colonic epithelial cells, which is also secreted. It has, therefore, the potential to mediate the sialyl-Lewis-mediated cellular interactions. Membrane-associated MUC2 would facilitate the interaction of tumor cells with sialyl-Lewis-ligands, while the soluble MUC2 would effectively inhibit the interaction. It may be also hypothesized that the increased density of sialyl-Lewis on mucins in tumor cells is likely to enhance the polyvalent interaction with a receptor.

ACKNOWLEDGMENTS

We thank Drs. P. X. Xing and I. F. C. McKenzie for providing us with mAb BC3 and Drs. Klaus Ley and Michael Netter for helpful discussions. The excellent technical assistance of Ulrike Dethlefs is gratefully acknowledged.

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